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# Molecular and Expression Analysis of the Negative Regulators Involved in the Transcriptional Regulation of Acid Phosphatase Production in *Saccharomyces cerevisiae*

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**The PHO80 and PHO85 gene products encode proteins necessary for the repression of transcription from the major acid phosphatase gene (*PHO5*) of *Saccharomyces cerevisiae*. The deduced amino acid sequences of these genes have revealed that PHO85 is likely to encode a protein kinase, whereas no potential function has been revealed for PHO80. We undertook several approaches to aid in the elucidation of the PHO80 function, including deletion analysis, chemical mutagenesis, and expression analysis. DNA deletion analysis revealed that residues from both the carboxy- and amino-terminal regions of the protein, amounting to a total of 21% of the PHO80 protein, were not required for function with respect to repressor activity. Also, 10 independent single-amino-acid changes within PHO80 which resulted in the failure to repress *PHO5* transcription were isolated. Nine of the 10 missense mutations resided in two subregions of the PHO80 molecule. In addition, expression analysis of the *PHO80* and *PHO85* genes suggested that the PHO85 gene product was not necessary for *PHO80* expression and that the *PHO85* gene was expressed at much higher levels in the cell than was the *PHO80* gene. Furthermore, high levels of PHO80 were shown to suppress the effect of a *PHO85* deletion at a level close to full repression. Implications for the function of the negative regulators in this system are discussed.**

Significant advances have been made concerning the regulatory mechanisms involved in activating as well as repressing transcription. Some of these mechanisms include the direct interaction of activators with defined promoter sequences, the interaction of repressors directly with activators, posttranslational modifications, feedback regulation, and many others (18, 19). The repressible acid phosphatase system of *Saccharomyces cerevisiae* provides an opportunity to study a system with two gene products required for repression.

In *S. cerevisiae*, acid phosphatase is produced predominately from the *PHO5* gene, which encodes a polypeptide necessary for balancing $P_i$ levels within the cell. Transcription of the *PHO5* gene is regulated in response to the level of $P_i$ available to the cell (2–5); therefore, *PHO5* is transcribed at high levels in media containing low amounts of $P_i$. Transcription of *PHO5* is repressed when cells are supplied with high amounts of $P_i$ in media. Several gene products are necessary for maintaining this balance and are involved in regulating *PHO5* transcription in response to the $P_i$ level (22).

The products of the *PHO81*, *PHO4*, and *PHO2* (*GRF10* or *BAS2*) genes are necessary for the activation of transcription of *PHO5*. PHO4 and PHO2 are constitutively produced at low levels and have been shown to bind specifically to DNA sequences within the *PHO5* promoter (15, 21, 30). In addition, PHO2 is involved in the transcriptional regulation of several additional genes in *S. cerevisiae* (1, 6). The PHO81 gene product appears to be regulated transcriptionally in response to $P_i$ levels in a fashion similar to that of *PHO5* (28). No function has been attributed to PHO81, but it is currently believed that PHO81 is necessary for relieving repression by direct interaction with the negative regulator PHO80 and/or PHO85 (22). Both the PHO80 and PHO85 gene products are required to maintain repression of *PHO5* transcription when cells are grown in media containing high $P_i$ levels, and *PHO80* and *PHO85* are constitutively expressed (16, 29). PHO85 encodes a protein with strong homology to known protein kinases (27) and has been implicated in the regulation of *PHO80* transcription (26).

Several characteristics of the PHO80 gene product are interesting with respect to its proposed function. (i) PHO80 is rich in serine, threonine, and tyrosine residues, which comprise 23% of the 293-amino-acid protein (16). Since it is proposed that phosphorylation plays a role in the regulation of acid phosphatase production at some step in the regulatory cascade, the presence of a large amount of serine, threonine, and tyrosine residues may play a role by composing substrates for posttranslational modification. (ii) PHO80 is highly basic, predominately within the carboxy-terminal half of the protein, with a net positive charge of +17 (16). Several transcriptional activators have been functionally dissected, and it has been determined that acidic domains within these proteins act as the transcriptional activation domains (10, 14). Although unprecedented, antagonism of an acidic activation region via interaction with basic amino acids provides a plausible means of repressing transcription.

The inclusion of a putative protein kinase as a negative regulator (PHO85) in the transcriptional control of acid phosphatase production is interesting in that the system is regulated by $P_i$ concentrations and also with respect to other genes in *S. cerevisiae* whose transcription is regulated directly or indirectly by a protein kinase. Although the SNF1 protein kinase of *S. cerevisiae* acts as a positive activator of transcription, this system may be analogous to the acid

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TABLE 1. Strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| YP98 | *MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 leu2-Δ1* | P. Hieter |
| YP102 | *MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1* | P. Hieter |
| 98/102 | *MATa*/α ura3-52/*ura3-52 lys2-801*/*lys2-801 ade2-101*/*ade2-101 trp1 trp1-Δ1*/*TRP1 his3-Δ200*/*HIS3 leu2-Δ1*/*leu2-Δ1* | P. Hieter |
| h-A | *MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 PHO80::LEU2* | This study |
| h-B | *MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 PHO80::LEU2* | This study |
| 98-85-TRP | *MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 leu2-Δ1 PHO85::TRP1* | This study |
| 242-281-1 | *MATa ura3-52 his3-Δ200 trp1-Δ1 ade2-101 lys2-801 pho81* | This study |
| D80-81 | *MATa*/α *ura3-52*/*ura3-52 trp1-Δ1*/*trp1-Δ1 ade2-101*/*ade2-101 lys2-801*/*lys2-801 his3-Δ200*/*his3-Δ200 leu2-Δ1*/*leu2-Δ1 pho81*/*pho81 PHO80::LEU2*/*PHO80::LEU2* | This study |

phosphatase system in that a cofactor (SNF4) is necessary for full function of the SNF1 protein kinase (7, 8). Similar phenotypes of PHO80 and PHO85 mutant strains suggest a possible interaction between these two factors to obtain a fully functional repressor.

To begin to elucidate the mechanism involved in the repression of transcription from the *PHO5* gene, we undertook a mutational analysis of *PHO80*. In addition, any interdependent regulation of *PHO80* and *PHO85* expression and function was examined.

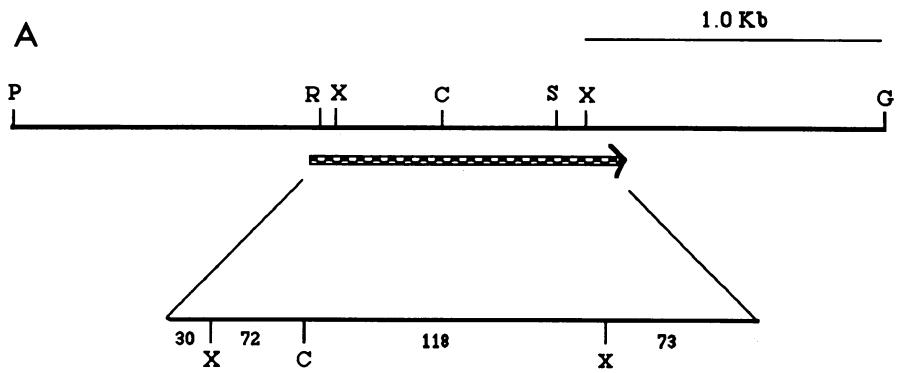
## MATERIALS AND METHODS

**Strains, media, and enzyme assays.** All yeast strains used in this study are listed in Table 1. Strains containing *PHO80* disruptions (h-A and h-B; Table 1) contained entire deletions of the genomic copy of *PHO80*. Yeast strains were grown in YPD medium supplemented with 2% glucose (24). Yeast strains harboring plasmids were grown in synthetic medium as described previously (24). Transformations of yeast cells were performed by the lithium acetate procedure described by Ito et al. (11). All *Escherichia coli* work was performed by standard procedures (17). Acid phosphatase and β-galactosidase levels were determined by methods described previously (9, 16).

**Deletion analysis.** All deletions were constructed with BAL 31 exonuclease (Bethesda Research Laboratories) as directed by the manufacturer. Carboxy-terminal deletions were constructed with plasmid YEp351-80CENTA, a derivative of YEp351 in which the *PHO80* gene, the proximal centromere (CEN XV), and a fragment containing the *TRP1* and *ARS1* genes were inserted. The plasmid was cleaved uniquely with *Bam*HI approximately 500 bp downstream from the carboxy-terminal end of the *PHO80* coding sequence. Bidirectional BAL 31 deletions were made, and either *Xba*I or *Spe*I linkers (New England BioLabs) containing translation stop codons in all three reading frames were inserted at the deletion endpoints. All deletions were sequenced by the dideoxy chain termination method of Sanger et al. (23) with oligonucleotides homologous to *PHO80* sequences. Amino-terminal deletions were generated from a unique *Eco*RI site 25 bp upstream of the initiation codon of *PHO80* (16). *Bam*HI linkers were inserted at deletion endpoints, and selected clones were sequenced to determine deletion endpoints. *Bam*HI fragments containing the deletion derivatives were cloned into a YCp50 derivative containing the *CYC1* promoter obtained from plasmid pLG669Z (provided by L. Guarente; 9). Deletion derivatives were ligated in-frame to the *CYC1* initiation codon and sequenced to confirm the reading frame. The function of the deletion derivatives was determined by transformation of the centromeric plasmids into a yeast strain (containing a disruption of the *PHO80* gene), and the level of acid phosphatase was assayed.

**Mutagenesis.** The selection scheme used to obtain *PHO80* mutations was as follows. Two centromeric plasmids were transformed into a diploid strain homozygous for both a *PHO80* disruption and a *pho81* mutation. The first plasmid was a *TRP1*-containing plasmid, p18-80, containing the entire *PHO80* gene. The second plasmid, p947-270 (a derivative of pBM947 [M. Johnston]), carried a *HIS3* reporter gene and a *URA3* selectable marker. The *HIS3* gene was fused to the *GAL1* core-promoter elements, including the TATA box, but was lacking the *GAL* upstream activating sequences (12, 13). The *GAL* upstream activating sequences were replaced by a *Bam*HI-*Cla*I fragment containing all of the *PHO5* upstream activating sequences (3). Selection for plasmid maintenance was initially achieved by growth in minimal medium lacking tryptophan and uracil. Cells (50 ml) were grown to the mid-log phase, washed with water, and suspended in 6 ml of 0.1 M $NaPO_4$ (pH 7.4). Two milliliters was treated with 35 μl of ethyl methanesulfonate (Sigma), 2.0 ml was untreated, and the cells were incubated at 37°C in a spinner wheel for 30 min. Cells were centrifuged, washed with 10 ml of 10% $Na_2S_2O_3$, washed with 0.1 M $NaPO_4$ (pH 7.4), and suspended in 2.0 ml of water. Cells were plated onto medium lacking tryptophan and uracil and incubated for 3 to 5 days at 30°C. Cells were replica plated to medium lacking tryptophan and histidine, with raffinose as a carbon source, and incubation was continued at 30°C. Colonies which grew in the absence of histidine were divided into grids on plates containing 5-bromo-4-chloro-3-indolyl-phosphate as a chromogenic substrate for acid phosphatase activity. Colonies expressing acid phosphatase were maintained on the yeast plates lacking only tryptophan to cure the cells of the *URA3*-containing plasmid. The yeast cells were collected and lysed, and total DNA was used to transform *E. coli* to isolate the *TRP1*-containing PHO80 plasmid. Plasmid DNA was used to transform strain h-A, and acid phosphatase expression was screened on solid medium as described above. Isolates retaining constitutive expression of acid phosphatase were retained for further analysis. Plasmids from these strains were sequenced along the entire length of the *PHO80* gene to determine the nature of the mutation.

**Blots.** Northern (RNA) blots were made as described previously (16, 25). For the quantitation of *PHO80* and *PHO85* transcripts, 1.0-kb fragments containing the entire coding regions of these genes were labeled and used to probe slot blots containing a plasmid bearing single copies of both the *PHO80* and *PHO85* genes to determine specific activity (the *PHO85*-specific probe used had 3.5-fold-higher activity than did the *PHO80*-specific probe used). RNA slot blots were probed simultaneously with these same probes. Western blots (immunoblots) were performed with a Bio-Rad immunoblotting kit with either alkaline phosphatase- or horseradish peroxidase-coupled secondary antibodies. The primary antibody was a monoclonal anti-β-galactosidase antibody used as directed by the manufacturer (Boehringer




| PLASMID | AMINO ACIDS DELETED | %APASE ACTIVITY |
|---|---|---|
| B C3-B | 0 | 6 |
| pB1453 | NO PHO80 | 100 |
| Δ866 | 4 | 9 |
| Δ848 | 11 | 6 |
| Δ815 | 21 | 11 |
| Δ780 | 32 | 9 |
| Δ751 | 43 | 12 |
| Δ734 | 47 | 12 |
| Δ725 | 51 | 92 |
| Δ669 | 70 | 92 |
| Δ637 | 80 | 93 |
| C CYC-20 | 0 | 0 |
| CYC-3 | 16 | 0 |
| CYC-59 | 23 | 62 |
| CYC-58 | 36 | 93 |
| CYC | NO PHO80 | 100 |

FIG. 1. (A) Partial restriction map of the PHO80 gene. X, *Xba*I; C, *Cla*I; P, *Pst*I; R, *Eco*RV; S, *Sac*II; G, *Bgl*II. The expanded region represents the *PHO80* coding region. Numbers indicate the numbers of amino acids between each restriction site. (B) Carboxy-terminal deletion analysis of PHO80. Percent acid phosphatase (APASE) activity was determined in high-$P_i$ medium and is expressed relative to that in a plasmid bearing no PHO80 molecule (pB1453). (C) Amino-terminal deletion analysis of PHO80; assays were done as for panel B.

Mannheim). Slot blots were made with the use of a Hybri-slot manifold (Bethesda Research Laboratories). All hybridizations were carried out as previously described (4), except that randomly primed probes were labeled with [$^{32}$P]dATP (New England Nuclear) with a Boehringer Mannheim random priming kit.

**β-Galactosidase fusions.** β-Galactosidase expression vectors of PHO80 fusions and mutant PHO80 fusions were constructed by first replacing the *PHO80* promoter with the *CYC1* promoter as in the deletion analysis described above. *CYC1* promoter-PHO80 derivates were ligated in-frame to plasmid YEP356R described previously (20). Amino-terminal fusions (CYC-N) were at amino acid 30 within PHO80, and carboxy-terminal fusions (CYC-X8) were at amino acid 279 within PHO80.

## RESULTS

**Deletion analysis of PHO80.** To aid in determining any functional significance of domains within PHO80, we undertook a deletion analysis from both the carboxy- and amino-terminal ends of the protein. A partial restriction map of the *PHO80* gene is shown in Fig. 1. Carboxy-terminal deletions were constructed as described in Materials and Methods with either *Xba*I or *Spe*I nonsense linkers to ensure proper termination of translation. The deletion derivatives were introduced into a strain containing a *PHO80*-disrupted allele (16) on a centromere-bearing plasmid. Amino-terminal BAL 31 deletions were constructed as described in Materials and Methods and fused in-frame to an ATG initiator codon downstream of the *CYC1* promoter on a centromeric plasmid. The resulting *CYC1* promoter-PHO80 fusions were introduced into the yeast strain described above. The ability of the *PHO80* deletions to repress *PHO5* transcription under high-$P_i$ growth conditions was assayed by measuring acid phosphatase activity.

A total of 21% of the PHO80 protein was found to be nonessential for function. Forty-seven amino acids could be deleted from the carboxy terminus of PHO80 with no effect on the ability of PHO80 to act as a repressor. If an additional four amino acids were deleted from the carboxy terminus, a nonfunctional PHO80 molecule resulted. Sixteen amino acids at the amino terminus of PHO80 were also found to be nonessential for function. A further deletion of seven amino acids from the amino terminus resulted in a PHO80 molecule able to repress transcription at a level only 40% that of a fully functional molecule. A deletion of 36 amino acids from the amino terminus resulted in a nonfunctional PHO80 protein. It is worth noting that the deletions which did not repress *PHO5* transcription (either carboxy or amino terminal) also failed to repress (*PHO5*) expression when placed on high-copy plasmids (versus the centromeric plasmids described above) (data not shown).

Although no significance can be attributed to potential phosphorylation sites through such a deletion analysis, truncation of a substantial amount of the highly basic carboxy-terminal region of PHO80 had no effect. Interestingly, PHO80 function was not lost until deletions from the carboxy-terminal region encroached upon the second highly basic region of PHO80.

Current models for the regulation of acid phosphatase production suggest an interaction of one or more of the negative regulators (PHO80 and/or PHO85) in this system with the proposed mediator, PHO81. It is feasible that deletion derivatives of PHO80 able to retain function (i.e., repress) may be unable to interact with the mediator and may result in a nonderepressible phenotype. However, when the largest deletions from the amino and carboxy termini which allowed function to be retained were analyzed under derepressed, low-$P_i$ conditions, no effect on derepression was detected. This result suggests that the regions of the protein which were deleted were unlikely to interact with the

TABLE 2. Missense mutations found in the *PHO80* gene

| Residue | Amino acid change | Frequency[a] |
|---|---|---|
| 1 | Met→Ile | 2 |
| 30 | Cys→Tyr | 3 |
| 38 | Leu→Phe | 2 |
| 41 | Arg→Gln | 3 |
| 130 | Ala→Thr | 1 |
| 136 | Asp→Asn | 2 |
| 148 | Gly→Glu | 2 |
| 148 | Gly→Arg | 1 |
| 149 | Gly→Glu | 1 |
| 172 | Pro→Leu | 3 |
| 229 | Gly→Asp | 1 |

[a] Number of times an individual mutation was isolated.

mediator in this system (data not shown). Additionally, we were unable to construct any functional PHO80 derivatives containing either internal in-frame deletions or small in-frame insertions at either of the two *Xba*I sites or the unique *Cla*I site (data not shown; see Fig. 1 for locations of restriction sites).

**Chemical mutagenesis of PHO80.** To further establish the functional significance of domains of PHO80, we performed mutagenesis with ethyl methanesulfonate as described in Materials and Methods. Our selection for single-amino-acid changes that created nonfunctional mutants of PHO80 was based on the ability of *PHO5* activators to derepress transcription under normally repressed (high-$P_i$) growth conditions. A total of 10 single-amino-acid changes within PHO80 were shown to allow acid phosphatase production under high-$P_i$ growth conditions (Table 2). We anticipated that our detection of *PHO80* mutations was saturated when identical mutations were found several times. No mutations which resulted in the loss of PHO80 function were detected within the promoter region of *PHO80*. Also, seven frameshift and three nonsense mutations were detected. Finally, aside from the initiator codon, no nonfunctional mutations were found within regions found to be nonfunctional via deletion analysis (see above).

The mutations detected fell into two general subregions of the PHO80 protein. The first encompassed amino acids 30 to 41, with three amino acid changes resulting in a nonfunctional PHO80 molecule. The second region contained a larger number of amino acids, ranging from amino acids 130 to 173, with six null mutations detected. Finally, a single-amino-acid change at amino acid 229 also resulted in a nonfunctional PHO80 molecule. Six of the 10 mutations either created a charged side group or altered the charge of the original amino acid. The remaining alterations were all nonconservative in that they involved changes from nonaromatic to aromatic or nonpolar to polar amino acids.

Because of the potential for alterations resulting in an unstable mutant protein, we designed an assay to detect the levels of expression of wild-type and mutant derivatives of PHO80 (see Materials and Methods). This was achieved by fusing PHO80 to β-galactosidase at amino acid 279 of the PHO80 protein. Expression of the fusion derivatives was placed under the control of the *CYC1* promoter, and the ability to complement a *PHO80* disruption strain was examined (Fig. 2A). The wild-type PHO80-LacZ fusion was able to complement the disruption. As expected, of the three mutant derivatives tested at random, none was able to complement the disruption (data not shown). Steady-state levels of the fusion proteins were analyzed for β-galactosidase activity and by Western blot analysis with an anti-β-galactosidase antibody (Fig. 2B). All three mutants contained comparable levels of the fusion peptide, as detected by Western blot analysis (Fig. 2B), and β-galactosidase activity (data not shown). Although the experiment analyzed only a subset of the mutations, the inability of the mutant PHO80 molecules to repress transcription cannot be ascribed to protein instability alone. We suggest that the majority of the mutations exerted their effect by amino acid alterations of structural domains of PHO80 required for repressor function. Finally, the levels of wild-type and mutant fusion polypeptides were unaffected by the addition

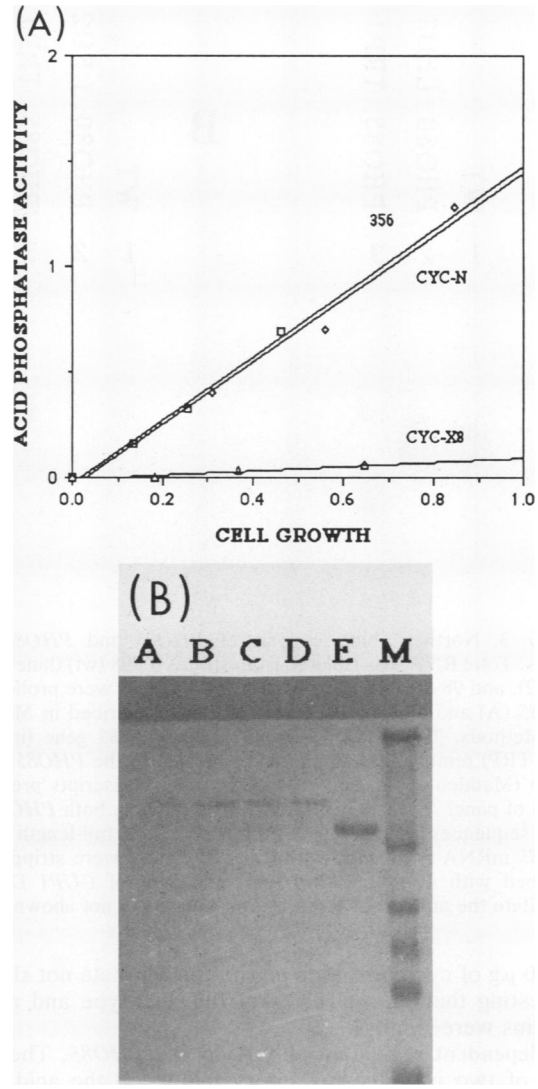



FIG. 2. (A) Plot of acid phosphatase activity versus cell growth in high-$P_i$ medium. Plasmids were transformed into strain h-A (Table 1) as described in Materials and Methods. YEP356R (356) (□) is a β-galactosidase-containing plasmid with no PHO80 sequences present, resulting in no expression of β-galactosidase (20). CYC-N (◇) and CYC-X8 (△) are PHO80–β-galactosidase fusions (see Materials and Methods). CYC-N is a PHO80–β-galactosidase fusion at amino acid 30 (out of 293) of PHO80. CYC-X8 is a PHO80–β-galactosidase fusion at amino acid 279 (out of 293) of PHO80. Both fusions are expressed from the *CYC1* promoter. (B) Western blot analysis of PHO80 fusions to β-galactosidase. Lanes: A, CYC-X8 (Arg-41→Gln); B, CYC-X8 (Pro-172→Leu); C, CYC-X8 (Ala-130→Thr); D, CYC-X8; E, CYC-N; M, molecular weight standards ($M_r$s, 180,000; 116,000; 84,000; 58,000; 48,500; and 36,500).

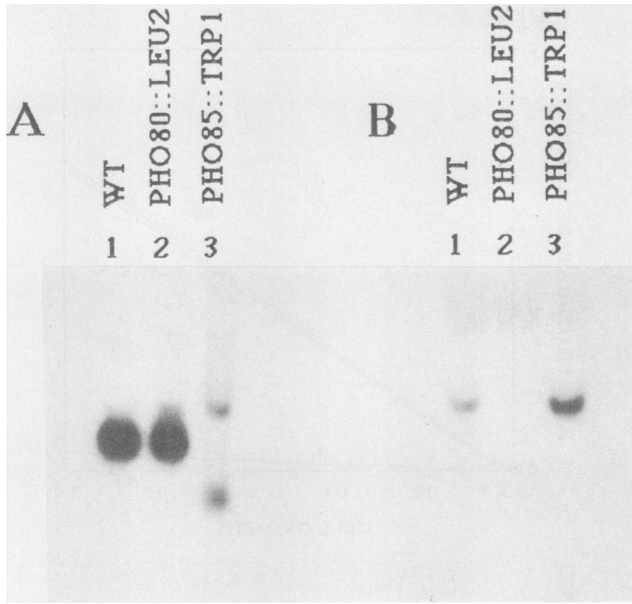



FIG. 3. Northern blot analysis of *PHO85* and *PHO80* transcripts. Total RNA was isolated from strains YP98 (wt) (lane 1), h-A (lane 2), and 98-85-TRP (lane 3) (Table 1). Filters were probed with *PHO85* (A) and *PHO80* (B) linear DNA as described in Materials and Methods. The partial deletion of the *PHO85* gene (in strain 98-85-TRP) removed 30 amino acids internal to the *PHO85* coding region (Madden et al., submitted). The two transcripts present in lane 3 of panel A represent transcripts containing both *PHO85* and *TRP1* sequences (data not shown). No detectable full-length mature *PHO85* mRNA is present in this strain. Filters were stripped and reprobed with a 700-bp *Xba*I-*Kpn*I fragment of *CUP1* DNA to quantitate the amount of RNA on the filter (data not shown).

of 100 μg of cycloheximide per ml for 1 h (data not shown), suggesting that the half-lives of the wild-type and mutant proteins were similar.

**Independent regulation of *PHO80* and *PHO85*.** The presence of two negative regulatory factors in the acid phosphatase regulatory cascade is a rather unique situation, and an understanding of any interdependence between these two regulatory factors is crucial to the elucidation of the transcriptional regulation of acid phosphatase production. It has previously been reported that *PHO80* and/or *PHO85* is expressed constitutively, regardless of the $P_i$ concentration present in the medium (16, 29). In addition, evidence has been presented suggesting that the expression of *PHO80* is dependent on the presence of an intact PHO85 gene product (26). We have examined this latter conjecture and have also studied the relationship between *PHO80* expression and *PHO85* expression.

The genomic copy of *PHO85* was disrupted (removing 89 bp from the coding sequence of *PHO85* and replacing these base pairs with *TRP1*; S. L. Madden, D. L. Johnson, and L. W. Bergman, submitted for publication), and total RNA was isolated from wild-type cells, a *PHO80* deletion strain, and the *PHO85* deletion strain. Northern blot analysis was performed by probing with *PHO80*- and *PHO85*-specific probes (see Materials and Methods). The disrupted strains did not produce full-length transcripts from the genes which were disrupted (Fig. 3). However, in disagreement with previous results (26), we were able to detect a full-length *PHO80* transcript in the *PHO85* disruption strain (Fig. 3B, lane 3). Furthermore, levels of a PHO80–β-galactosidase fusion protein were unaffected by deletion of the *PHO85* gene (Table 3). Wild-type levels of the *PHO85* transcript were produced in the *PHO80* disruption strain (Fig. 3A, lane 2), and the levels of the PHO85–β-galactosidase fusion protein were unaffected by deletion of the *PHO80* gene (Table 3). We conclude that no interdependent transcriptional regulator occurs between *PHO80* and *PHO85*.

TABLE 3. β-Galactosidase activities of PHO80-LacZ or PHO85-LacZ fusion proteins in various yeast strains

| Fusion[a] | Strain | β-Galactosidase sp act (U/mg of protein) |
|---|---|---|
| R80-NZ | YP98 | 31 |
| ER85 | YP98 | 287 |
| R80-NZ | h-A (*PHO80*::*LEU2*) | 36 |
| ER85 | h-A (*PHO80*::*LEU2*) | 229 |
| R80-NZ | 98-85-TRP (*PHO85*::*TRP1*) | 55 |
| ER85 | 98-85-TRP (*PHO85*::*TRP1*) | 400 |

[a] The fusion of PHO80 to β-galactosidase occurred at amino acid 30 of PHO80. The fusion of PHO85 to β-galactosidase occurred at amino acid 79 of PHO85. Both of the plasmids were constructed with YEP356R (20).

**Quantitation of *PHO80* and *PHO85* mRNA and protein.** Although *PHO80* and *PHO85* have been shown to be transcribed and translated constitutively (16, 29), we wished to quantitate any differences in the expression of *PHO80* and *PHO85* at the mRNA and protein levels. Fragments from both the *PHO80* and the *PHO85* genes were radioactively labeled independently and used to probe RNA slot blots along with slot blots containing a DNA plasmid constructed to include both the *PHO80* and the *PHO85* gene to differentiate between the specific activities of the two probes. RNA slot blot results are shown in Fig. 4. Blots were initially probed with either the *PHO80*- or the *PHO85*-specific probe, followed by washing and rehydridization with a probe specific for the *CUP1* gene to account for any fluctuation in the RNA quantities applied to the filters (see Materials and Methods). Using densitometric scanning and adjusting for differences in the specific activities of the *PHO80*- and *PHO85*-specific probes, we found that the ratio of steady-state *PHO85* transcripts to steady-state *PHO80* transcripts was approximately 6:1.

To further corroborate these results suggestive of a significantly higher level of PHO85 than of PHO80 in cells, we constructed β-galactosidase fusions to PHO80 and PHO85 at amino acids 30 (PHO80) and 79 (PHO85). β-Galactosidase activity and Western blot analyses were performed on steady-state cultures of wild-type strains transformed with multicopy plasmids containing the fusions. The PHO85–β-galactosidase fusion was produced in wild-type cells (YP98) at approximately 10-fold-higher levels than was the PHO80–β-galactosidase fusion (Table 3). The fusion plasmid copy numbers in each strain were determined by DNA dot blot analysis and found to be equivalent (data not shown). Western blot analysis with an anti-β-galactosidase antibody confirmed the 9- to 10-fold-higher level of the PHO85–β-galactosidase fusion polypeptide (data not shown). We conclude that *PHO85* is expressed at 6- to 10-fold-higher levels than is *PHO80* and that these levels are likely to be important for any interactions needed for proper regulation of acid phosphatase production.

**Suppression of a *PHO85* deletion by overexpression of *PHO80*.** A previous study showed that overexpression of the PHO80 gene product in a wild-type background resulted in

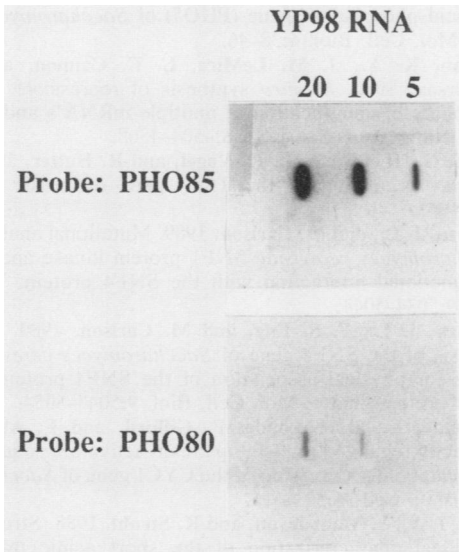



FIG. 4. Quantitation of *PHO85* and *PHO80* transcripts by slot blot analysis. Total RNA (20, 10, and 2 μg) isolated from strain YP98 was added to the filters (starting at the left and proceeding to the right) and probed with either a *PHO85*-specific probe or a *PHO80*-specific probe as described in Materials and Methods. Films were scanned and quantitated with an E-C densitometer. Filters were stripped and reprobed with a 700-bp *Xba*I-*Kpn*I fragment of *CUP1* DNA to quantitate the amount of RNA on the filter (data not shown). The *PHO85*-specific probe had a specific activity 3.5-fold higher than that of the *PHO80*-specific probe. Each probe was 1.0 kb in length and contained the entire coding region of the respective gene.

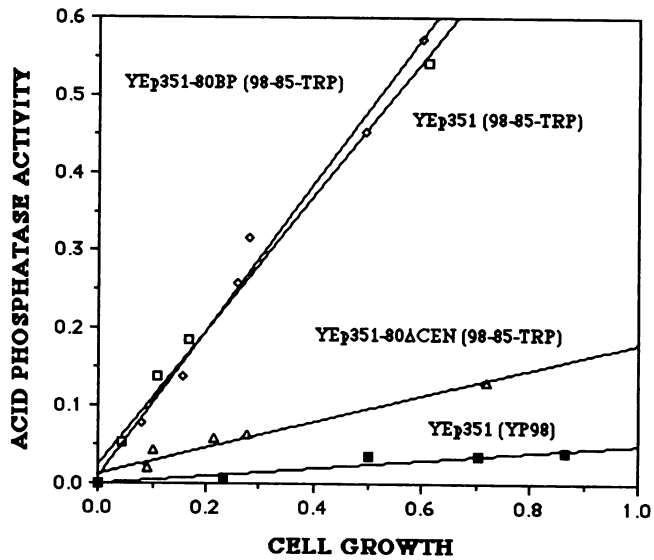



FIG. 5. Effect of *PHO80* overexpression in a *PHO85* deletion strain. Plasmids were transformed into 98-85-TRP (□, △, and ◇) or YP98 (■), and acid phosphatase activity was determined. Symbols: □ and ■, YEp351; ◇, YEp351-80BP, containing a centromere and *PHO80*; △, YEp351-80ΔCEN, containing *PHO80* but lacking a centromere. YEp35180BP and YEp35180ΔCEN have been described previously (16).

repression of acid phosphatase production under the normally derepressed conditions of low-$P_i$ concentrations (16). This result suggested that PHO80 is able to function as a repressor in the derepressed state and points to a delicate balance of *trans*-acting factors necessary for proper transcriptional regulation. Therefore, we wanted to examine any effect of overexpression of *PHO80* in strains with a deletion of the *PHO85* gene. Overexpression of *PHO80* in a *PHO85*-disrupted background resulted in the production of repressed levels of acid phosphatase to a level slightly greater than that in the presence of full repression (Fig. 5). This result suggests that PHO85 is not directly responsible for repression or that some other factor is able to substitute for PHO85 in the cell (see below).

## DISCUSSION

In the present study, we sought to determine the regions of PHO80 necessary for repressor function and to examine any relationship which may exist between *PHO80* expression and *PHO85* expression. Deletion analysis revealed that 21% of the PHO80 protein is dispensible for full repressor function. The majority of the nonfunctional sequences reside within the highly basic carboxy-terminal region of the protein. Forty-seven amino acids from the carboxy terminus (16% of the 293-amino-acid protein) were shown to be dispensible to retain function of the PHO80 protein. A plot of net charge versus residue number of PHO80 suggested that there are two highly basic regions present in the carboxy-terminal half of the PHO80 protein. Our deletion analysis showed that the most carboxy-terminal basic region can be deleted without any loss of function. Function is lost, however, when the next basic region starts to be deleted. The significance of basic nature to the function of PHO80 is presently not clear. However, two possible functions are plausible. (i) Several transcriptional activation factors contain acidic residues shown to provide a functional activation domain (10, 14). PHO4 and PHO2 also contain acidic stretches of amino acids, and the acidic region of PHO4 has recently been shown to be important for transcriptional activation (21). The interaction of basic sequences within a protein with acidic activating regions could provide a means for repressing transcriptional activation. (ii) The basic PHO80 protein may interact with DNA sequences within the PHO5 promoter. With PHO80 and PHO85 acting as corepressors in acid phosphatase production, a requirement for PHO80 interaction with PHO85 may allow for proper specificity and function of the PHO85 protein kinase.

Chemical mutagenesis of PHO80, resulting in single-amino-acid changes which prevented proper repressor function, yielded 10 unique, nonfunctional mutations. Nine of the 10 mutations fell into two general regions of the PHO80 protein. Analysis of steady-state expression of several of the mutants ruled out the possibility that the phenotypes of the mutations are strictly due to instability or the inability to express the mutant proteins. The clustering of mutations in specific subregions of the PHO80 protein suggested that these regions are important for any interactions required for repressor function.

The determination of any expression differences between the two negative regulatory factors may provide clues to the potential interaction of these factors or of other factors involved in regulating acid phosphatase expression. We have shown that, although both PHO80 and PHO85 are produced constitutively, *PHO85* is expressed at levels 6- to 10-fold higher than is *PHO80*. It should be noted that increased levels of the PHO80 gene product (resulting from either placement of the gene on a high-copy-number plasmid or placement of *PHO80* under the control of the highly induc-

ible *GAL1* promoter) alter the normal regulation of acid phosphatase production (16). If PHO80 and PHO85 interaction is required for proper repression, one needs to determine why there is such a difference in expression. The interaction of PHO85 and PHO80 in a proportion equivalent to the expressed levels of the two proteins is unlikely because of the ratio of these proteins in the cell. PHO85 may be involved in the phosphorylation of more than one substrate, and the requirement for PHO80 may be limited to a subset of these substrates. Finally, PHO85 may not interact with PHO80 but may require a previous interaction of PHO80 with the phosphorylated substrate(s) or vice versa. Our results showing nearly complete compensation for a *PHO85* deletion by overexpressed *PHO80* suggest an indirect role of PHO85 in repression. These results are in direct contrast to those in another yeast regulatory system with a protein kinase (SNF1) and a cofactor (SNF4) partially responsible for kinase function (7, 8). In this case, it appears that SNF1 kinase function is directly responsible for transcriptional activation (7, 8), whereas in acid phosphatase regulation, suppression of a *PHO85* deletion by increased levels of *PHO80* suggests a more direct role of PHO80 than of PHO85 protein kinase in repression. One possibility is that PHO85 functions to phosphorylate a positive activator (e.g., PHO4), which then allows for an increase in PHO80 affinity for and subsequent transcriptional repression of the PHO4 protein. Compensation of a *PHO85* deletion by increasing amounts of *PHO80* is not a result of PHO85 involvement in *PHO80* transcription, as suggested previously (26). Our *PHO85* deletion strain contained amounts of *PHO80*-specific transcripts similar to those contained in a wild-type strain.

In acid phosphatase regulation, we envision that high phosphate concentrations indirectly increase the activity of PHO85, which subsequently increases the affinity of PHO80 for one or more of the positive factors. Interestingly, *PHO85* disruption strains showed aberrant growth on several carbon sources, suggestive of a possible role of the PHO85 gene product in other regulatory systems in the cell (Madden et al., submitted). Presently, we are attempting to elucidate the interactions occurring between factors necessary for activation as well as repression of *PHO5* transcription.

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